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(54) Title: NOVEL MODIFIED PF4 COMPOSITIONS AND METHODS OF USE

(57) Abstract

The subject invention pertains to the use of modified PF4 to inhibit angiogenesis. The modified PF4 has utility for treating angiogenic diseases and for the inhibition of endothelial cell proliferation. Also, the subject invention concerns modifications of PF4 which extend the half-life and facilitate the targeting of the biological activity of PF4 to specific locations. Furthermore, PF4 itself can be used to target the activities of other molecules to locations of antiogenesis and endothelial cell proliferation.

# DESCRIPTION

# NOVEL MODIFIED PF4 COMPOSITIONS AND METHODS OF USE

## Cross-Reference to Related Applications

This application is a continuation-in-part of co-pending application Serial No. 08/149,104, filed November 5, 1993; which is a continuation-in-part of application Serial No. 08/110,477 filed August 23, 1993; which is a continuation of application Serial No. 07/731,222, filed July 15, 1991, now abandoned, which is a continuation-in-part of application Serial No. 07/376,333, filed July 6, 1989, now U.S. Patent No. 5,112,946.

## Background of the Invention

Angiogenesis, the development of new capillary blood vessels, is an important process in the developing fetus and growing human. However, in healthy adults, angiogenesis occurs significantly only during wound healing and in the menstrual cycle.

It is now widely recognized that much of the angiogenic activity occurring in adults is pathological in nature. For example, proliferation of vascular endothelial cells and formation of new capillaries is essential for growth of solid tumors beyond a few cubic millimeters in volume (Folkman *et al.* [1983] *Ciba Found. Symp.* 100:132-149). We now understand that developing tumors secrete growth factors which stimulate neighboring endothelial cells to divide and migrate toward the tumor.

In addition to growth of solid tumors, other conditions involving angiogenic dysfunctions include diabetic retinopathy, retrolental fibroplasia, neovascular glaucoma, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, hemangiomas, and Kaposi's Sarcoma. These, and other such conditions, are now widely recognized as diseases possessing characteristics of dysregulated endothelial cell division and capillary growth. These conditions along with growth of solid

tumors are collectively referred to as "angiogenic diseases" (Folkman, J., M. Klagsbrun [1987] *Science* 235:442-447).

In addition to angiogenic diseases, there are other conditions where endothelial cell proliferation is pathological or, at least, unwanted. For example, endometriosis is characterized by the abnormal proliferation and positioning of certain endothelial cells which normally line the inner wall of the uterus. Control of the angiogenic process could help to prevent or alleviate endometriosis. Also, prevention of endothelial cell growth in the uterus could be a means of birth control.

Endothelial cell growth is associated with wound healing. This growth is undesirable during extended surgical proceedings and where excessive scar formation may occur. Therefore, a means of controlling endothelial cell proliferation would help prevent or reduce unwanted scar formation.

The mechanism of angiogenesis and endothelial cell proliferation has not been completely characterized. It has been established that mast cells accumulate at a tumor site before new capillary growth occurs; however, mast cells alone cannot initiate angiogenesis. Heparin, a mast cell product, has been shown to significantly stimulate the capillary endothelial cell migration which is necessary for angiogenesis (Folkman, J. [1984] "Angiogenesis: Initiation and Modulation," In *Cancer Invasion and Metastasis: Biologic and Therapeutic Aspects*, G.L. Nicolson and L. Milas, eds., Raven Press, New York, pp. 201-208).

Several substances are known to have the capability of inhibiting endothelial cell growth *in vitro*. One of the most extensively studied inhibitors of endothelial cell growth is protamine, which is a protein found only in sperm. Clinical experiments with protamine have not been pursued because of the toxicity associated with protamine injection. At least two other compounds have been studied in regard to their heparin-binding activity: platelet factor 4 (PF4) and major basic protein. Major basic protein has demonstrated heparin-binding activity but is of little practical utility because of its high toxicity.

Platelet factor 4 is a well-known protein which has been completely sequenced (Deuel, T.F., P.S. Keim, M. Farmer, R.L. Heinrikson [1977] *Proc. Natl. Acad. Sci. USA* 74(6):2256-2258). It is a 70-residue secretable platelet protein with a molecular weight of approximately 7.8 kD. Although there were early reports that certain PF4

compositions may have anti-angiogenic activity (Folkman [1984], *supra*), the prior art did not disclose any clinical utility for PF4.

Compositions which contain a compound described as "oncostatin A," which appears to be the same, or similar to, native PF4, have been implicated as affecting the growth of tumors (U.S. Patent Nos. 4,645,828 and 4,737,580; both issued to Twardzik *et al.*). However, the effects reported in these patents pertain to slowly growing human cancer cells in immunodeficient mice. The results of these experiments cannot be reliably extrapolated to predict the effect on rapidly growing tumors which are native to the host animal. Furthermore, the experiments reported in these patents in no way predict or disclose any angiostatic properties. Also, the preparations used by Twardzik *et al.* were purified from platelets and likely contained a mixture of proteins. These purified platelet compositions would be expected to contain other biologically-active proteins including compounds such as TGF-β which are known to inhibit angiogenesis.

Various peptides from PF4 have been purified and their properties studied. It is known that the C-13 peptide of PF4 is chemotactic for neutrophils and monocytes (Deuel, T.F., R.M. Senior, D. Chang, G.L. Griffin, R.L. Heinrikson, E.T. Kaiser [1981] *Proc. Natl. Acad. Sci. USA* 78:4585-4587; Osterman, D.G., G.L. Griffin, R.M. Senior, E.T. Kaiser, T.H. Deuel [1982] *Biochem. and Biophys. Res. Comm.* 107(1):130-135). It is significant to note that the infiltration of monocytes would be expected to stimulate the proliferation and migration of local endothelial cells by the secretion of angiogenic factors. Thus, peptides of PF4 could be expected to stimulate, rather than inhibit, angiogenesis.

There is a significant and very long-standing need to locate an effective and non-toxic inhibitor of angiogenesis and endothelial cell proliferation. Angiogenesis plays a major role in the initiation and progression of widespread catastrophic illnesses, including cancer. An effective, non-toxic agent which can be administered locally and/or systemically to treat these illnesses would be highly advantageous and has long eluded identification.

The localization of angiogenesis and endothelial cell proliferation, as well as the determination of the margin between normal tissue and tumor (or other site of angiogenesis) can be useful for surgical, radiotherapeutic, and chemotherapeutic approaches to control or eliminate the unwanted angiogenesis. For example, it is well

established that the successful delivery of magnetic resonance contrast agents, radionuclides, or other detectable agents for imaging can contribute to the more precise localization of tumor boundaries. Monoclonal antibodies prepared against tumors have been proposed for use in the past as effective carrier molecules for the delivery of contrast and radionuclide agents. However, the use of such monoclonal antibodies can be accompanied by disadvantages. For example, antibodies are very large molecules that also can carry cross-reactive antigenic determinants that may immunoreact in undesirable ways. Furthermore, the use of monoclonal antibodies that bind with particular tumor-specific antigens can have an important limitation of being too specific. Due to their specificity, these antibodies may not recognize many unwanted neovascularization sites. Thus, a monoclonal antibody specific to a particular tumor agent may be effective in directing an imaging agent to that particular type of tumor, but other sites of neovascularization may go undetected. Furthermore, the tumor to which the antibody is specifically directed may not be reliably detected if the antigen recognized by the antibody is not exposed or otherwise is inappropriately presented. Therefore, a more reliable means for detecting sites of angiogenesis is needed.

In addition to monoclonal antibodies, various synthetic polypeptides, such as polylysine which selectively binds to tumor cells, have been considered for use as carrier agents for therapeutic or imaging agents. See, for example, U.S. Patent No. 5,230,883. Unfortunately, such polypeptides can be highly toxic and, therefore, impractical for clinical use.

A need exists for reliable, safe methods and materials for the localization, targeting, and treatment of sites of angiogenesis and endothelial cell proliferation and for complexes that can be used in such methods. Ideally, such methods and materials could detect a broad range of angiogenic sites.

## Brief Summary of the Invention

The subject invention relates to diagnostic and therapeutic compositions obtained through chemical modifications of platelet factor 4 (PF4) or recombinant PF4 (rPF4). As used herein, the term "PF4" refers both to PF4 purified from platelets and to recombinant PF4 unless otherwise noted. According to the subject invention, conjugates comprising PF4 can be made by chemically attaching a second entity to the

PF4. The subject invention further relates to methods for using the PF4 conjugates described herein.

The compositions and methods of the subject invention are particularly useful in the detection and treatment of tumors and other sites or areas of unwanted neovascularization. The compositions and methods of the subject invention can also be used to locate normal angiogenesis such as what may occur during pregnancy or during the formation of scar tissue. The compositions and methods of the subject invention are also useful for the rapid, accurate, and safe detection of sites of angiogenesis and/or endothelial cell proliferation. Because PF4 is non-toxic, non-immunogenic, and can be used systemically, the compositions and methods of the subject invention can be used to safely locate and treat widely disseminated sites of neovascularization.

The subject invention is described herein with respect to two primary embodiments. First, applicants have discovered that PF4 can be conjugated to a variety of entities and surprisingly retain its advantageous angiostatic properties. Therefore, in one embodiment of the subject invention, PF4 can be conjugated to a second entity which will target the activity of PF4 to a particular location where angiostatic activity is needed. Molecules which can be used in this manner to direct the activity of the PF4 include, but are not limited to, monoclonal antibodies, polyclonal antibodies, carrier proteins, carbohydrate lectins, cell receptor molecules, single chain antigen-binding proteins, and other binding protein sequences. Also, according to this embodiment of the subject invention, PF4 can be conjugated to molecules in order to prolong the half-life of the PF4 in the biological system. In a preferred mode, a polymer can be conjugated to PF4 to increase the circulating half-life of the PF4. The polymer may be, for example, a polyamino acid such as polyglutamate, a polysaccharide such as dextran, or a biocompatible polymer such as polyethylene glycol (PEG).

In a second primary embodiment of the subject invention, applicants have discovered that PF4 preferentially binds to sites of endothelial cell proliferation and angiogenesis. Therefore, PF4 can itself be used as a targeting agent and be conjugated to any of a variety of entities which are desired to be selectively and accurately delivered to sites of angiogenesis or endothelial cell proliferation. Thus, in this embodiment, PF4 is the molecule which directs the activity of a second entity to a

specific location. For example, PF4 can be conjugated to toxins which will kill tumor cells. Also, PF4 can be conjugated to imaging agents which facilitate the detection and visualization of sites of angiogenesis and endothelial cell proliferation. This embodiment is based on applicants' discovery that PF4 is transported systemically through the body to areas of angiogenesis including new proliferating vascular beds. For example, this embodiment can be used to facilitate the accurate visualization of tumors and other sites of angiogenesis. PF4 can also be used to detect sites of normal neovascularization such as may occur in pregnancy or during scar tissue formation. One of the advantages of using PF4 as the targeting agent is that it detects a wide range of sites of angiogenesis and it can safely be used systemically in such a procedure without toxicity.

In this second primary embodiment, PF4 can be conjugated with a second entity which can be detected by means known to those skilled in the art. This second entity can be, for example, a fluorescent compound or a radioisotope. The PF4 conjugate comprising the detectable second entity is then administered such that the PF4 can be biologically transported to the location at which knowledge of angiogenesis is desired. Advantageously, this transport can occur systemically.

Using the teachings provided herein, a person of ordinary skill in the art will be able to utilize standard chemical procedures to produce a variety of PF4 conjugates. For example, PF4 can be modified through its free amino groups with fluorescein-isothiocyanate (FITC) and retain the capability of inhibiting angiogenic activity and endothelial cell proliferation. Modification with FITC also makes it possible to visualize sites of angiogenesis and endothelial cell proliferation. Many other agents in addition to FITC can also be used for this purpose. As described in detail below, similar modifications can be made with PF4 analogs, mutants, or fragments.

One embodiment of the subject invention concerns the discovery of a particular region of the PF4 molecule at which conjugation can occur without losing biological activity. Specifically, the approximately 15 amino acids at the N-terminus of the full-length PF4 molecule has been discovered to be a preferred location for conjugation. The discovery of this region of the PF4 molecule, referred to herein as the "Preferred Conjugation Site," or "PCS," is an important aspect of the subject invention because it facilitates the production of PF4 conjugates having a broad range of highly useful biological activities. These biological activities include the angiostatic properties of

PF4 and the ability of PF4 to bind to proliferating endothelial cells, and these activities include a wide variety of desired biological activities which are provided by the molecule which has been conjugated to the PF4.

## Brief Description of the Drawings

**Figure 1** shows the inhibition of angiogenesis resulting from treatment with rPF4 and various related peptides.

**Figure 2** compares the amino acid sequence of rPF4 with rPF4-241.

**Figure 3** depicts the α-helical configurations of rPF4 and rPF4-241.

**Figure 4** compares the inhibition of angiogenesis resulting from treatment with rPF4 and rPF4-241.

**Figure 5** shows inhibition of human endothelial cell proliferation by rPF4 and rPF4-241.

**Figure 6** compares the inhibition of human umbilical vein endothelial cell proliferation resulting from treatment with rPF4 or rPF4-241.

**Figure 7** shows the ability of rPF4 to inhibit tumor growth.

**Figure 8** depicts the possible chemical structure of the C-terminal end of FrPF4.

**Figure 9** shows inhibition of human endothelial cell proliferation by FrPF4.

**Figure 10** shows inhibition of human endothelial cell proliferation by FrPF4-241.

**Figure 11** shows inhibition of tumor growth by FrPF4.

**Figure 12** shows activity of PF4 and PF4 conjugated to glycine (Gly-PF4) in the HUVEC proliferation assay.

**Figure 13** shows activity of PF4 and a PEG-PF4 conjugate in the HUVEC proliferation assay.

**Figure 14** shows the activity of PF4 and a biotin-PF4 conjugate in the HUVEC proliferation assay.

**Figure 15** shows the effect of intravenous administration of PEG-rPF4 or rPF4 on lung metastasis formation as measured by the total number of lung metastases.

**Figure 16** shows the effect of intravenous administration of PEG-rPF4 or rPF4 as measured by lung weight.

**Figure 17** shows inhibition of tumor growth achieved by intradermal injection of PEG-rPF4 or rPF4 as compared to controls.

**Figure 18** shows pharmacokinetics of PEG-rPF4 compared to rPF4 after intravenous administration to Sprague-Dawley rats.

**Figure 19** shows the biodistribution of intravenously administered PEG-rPF4 as compared to rPF4.

## Brief Description of the Sequences

**SEQ ID NO. 1** is the amino acid sequence of C-13-241.

**SEQ ID NO. 2** is the mutant sequence designated rPF4-211.

**SEQ ID NO. 3** is the mutant sequence designated rPF4-231.

**SEQ ID NO. 4** is the mutant sequence designated rPF4-241.

**SEQ ID NO. 5** is the mutant sequence designated rPF4-302.

**SEQ ID NO. 6** is the mutant sequence designated rPF4-303.

**SEQ ID NO. 7** is the mutant sequence designated rPF4-307.

**SEQ ID NO. 8** is the mutant sequence designated rPF4-308.

**SEQ ID NO. 9** is the mutant sequence designated rPF4-315.

**SEQ ID NO. 10** is a generic sequence formula according to the subject invention.

**SEQ ID NO. 11** is a more detailed sequence formula for practicing the subject invention.

## Detailed Description of the Invention

The subject invention concerns the discovery that PF4, rPF4, and fragments and mutants of these compounds can be chemically modified to create conjugates with highly desirable characteristics. For example, chemical modification of PF4, its fragments, and its mutants, has resulted in the discovery of compounds which show surprising ability to inhibit endothelial cell proliferation and angiogenic activity and they preferentially bind to proliferating endothelial cells.

For brevity, throughout the specification and claims, applicants refer to PF4. This is intended to include recombinant PF4 (rPF4), as well as PF4 purified from its natural source. Such references to PF4 should also be understood to refer to variants, mutants, and fragments as described herein. Also, as used herein, reference to

conjugation of PF4 to a second entity includes direct conjugation and indirect conjugation such as through a binder or chelation.

In one embodiment of the subject invention, PF4 is conjugated to entities which enhance, direct, or prolong the biological activity of PF4. For example, PF4 may be conjugated to molecules that specifically recognize sites of unwanted angiogenesis. Examples of such molecules are monoclonal antibodies and cell receptors which direct the angiostatic PF4 activity to a specific location such as a tumor. As another example, PF4 may be conjugated to molecules such as polyethylene glycol (PEG), which will prolong its half-life in the circulation of a treated animal.

In a second primary embodiment of the subject invention, PF4 is itself used to direct a second entity to certain locations within the body of an animal or *in vitro*. This embodiment arises from applicants' discovery that PF4 preferentially binds to sites of endothelial cell proliferation characteristic of angiogenesis. Because PF4 binds to these sites, it can be used to direct other molecules with useful properties to these sites. Therefore, although PF4 is itself a potent inhibitor of angiogenesis, it can be conjugated with other angiogenesis inhibitors which can result in enhanced or expedited activity. For example, PF4 can be conjugated with toxins which destroy tumor cells, thereby producing an enhanced effect when used with PF4 which itself inhibits tumor growth. Such a combination of PF4 with another active ingredient has the advantage of not only targeting these activities specifically to sites of neovascularization, but also such a combination can create a synergistic antitumor effect through the combined activities of the components of the conjugate.

A further illustration of the second embodiment of the subject invention is the use of PF4 conjugates in diagnostic procedures whereby the PF4 is used to direct a detectable label to sites of angiogenesis or cellular proliferation. These sites of angiogenesis can be pathological, as in the case or tumors, or can be normal, such as in the case of pregnancy or scar tissue formation. For example, PF4 may be conjugated to radioisotopes or fluorescent compounds which can readily be detected using procedures which are well known to those skilled in the art.

PF4 conjugates as angiogenesis inhibiting agents. The PF4 conjugates of the subject invention can be used in the treatment of angiogenic diseases. As used herein,

the term "angiogenic disease" refers to pathological events such as growth of solid tumors, and other conditions involving angiogenic dysfunctions including diabetic retinopathy, retrolental fibroplasia, ocular neovascularization, corneal neovascularization (including that which is induced by trauma, infection or irritation), neovascular glaucoma, macular degeneration, psoriasis, angiofibromas, immune and non-immune inflammation (including rheumatoid arthritis), capillary proliferation within atherosclerotic plaques, unwanted scar tissue formation, hemangiomas, and Kaposi's sarcoma. The PF4 conjugates described herein for the treatment of angiogenic diseases include conjugates of PF4 fragments, analogs, and variants.

The types of solid tumors that can be treated by PF4 conjugates of the subject invention include all types of lung tumors, including small cell lung carcinoma; tumors of the breast, colon/rectum, prostate, head and neck, stomach, bladder, kidney, pancreas, liver, ovary, and uterus; sarcomas; melanoma and other metastatic skin cancers; nonmetastatic skin cancers (e.g., Kaposi's sarcoma, basal cell carcinoma); and tumors of the brain.

The types of tumors that can be treated using the compositions and methods of the invention include tumors that are not surgically accessible and/or are resistant to chemotherapy and radiation therapy. Advanced invasive malignancy, with or without surgery, can be treated using the compositions and methods of the invention. The invention can be used as an adjuvant therapy following surgical resection and can be used to treat known metastatic disease and nonmetastatic cancer.

Treatment of the tumors and diseases described above can be either systemic, regional, or local (intralesional), depending upon the type and severity of the disease as well as the accessibility of the disease site. Systemic treatment includes intravenous bolus injections and infusions, subcutaneous injections, implants, refillable reservoirs, and sustained release depots and intramuscular injections. Regional treatment includes intraarterial for the treatment of primary liver tumors and liver metastases, and for the treatment of kidney, brain, and pancreatic tumors. Regional intraperitoneal treatment can be used for the treatment of tumors of the ovary. Local treatment can be used for tumors of the brain, uterus, bladder, head, and neck, for Kaposi's sarcoma and other nonmetastatic skin cancers, for metastatic skin cancer once dissemination precludes further surgical excision, and for colon and rectal cancer.

The PF4 conjugates of the subject invention can be administered as an alternative to known procedures such as surgery, chemotherapy, or radiation therapy. Also, the PF4 conjugates can be used concurrently with, or following, such procedures in order to enhance the effectiveness of these procedures by, for example, preventing further growth of any tumor tissue which may remain after an initial surgical, chemical, or radiation treatment.

Conjugation of PF4 to targeting agents specific to certain sites of neovascularization can be used to increase the accumulation of PF4 at the site where inhibition of angiogenesis is desired. These targeting agents are preferably specific for the type of tissue or tumor for which PF4 activity is desired. Specific examples of such targeting agents include proteins, antibodies, and binding fragments of antibodies. For example, other protein sequences which are known to bind to proliferating endothelial cells can be conjugated to PF4 according to the subject invention. Examples of proteins which bind to proliferating cells include tumor necrosis factor (TNF), transforming growth factor-β (TGF-β), antibodies to tumor-specific antigens, basic fibroblast growth factor (bFGF), prostate-specific antigen (PSA), and chorioembryonic antigen (CEA). Such a conjugate will direct the PF4 activity in a highly specific manner to a precise location where angiostatic activity is desired. Also, carbohydrate lectins are known to be specific for certain tumors. Examples of such specificities are galactose for melanoma, sarcoma, colon carcinoma, and Hodgkin disease; mannose for teratocarcinoma; glucose for colon carcinoma; and L-fucose for teratoma.

Also, conjugation of biocompatible polymers to PF4 according to the methods of the subject invention can be used to enhance circulation half-life of PF4 in the blood. This process can also be used to alter the biodistribution of the protein according to the type of polymer used. Specific examples are the use of polyethylene glycol (PEG), polyvinyl alcohol, and polyurethane.

One specific embodiment of the subject invention is the use of PF4 conjugates to treat diseases involving ocular neovascularization. These diseases can be very serious, leading in some cases to blindness. PF4 is highly advantageous in that it can be used to effectively treat these conditions without toxicity. The clinical utility of PF4 as a treatment for angiogenic diseases of the eye has been determined using two

different animal models. The first model simulates angiogenic diseases of the cornea and the second model simulates angiogenic diseases of the retina. These models are predictive of clinical utility for treating diseases involving neovascularization of the retina or cornea or other part of the eye (collectively referred to as ocular neovascularization).

The assay used to determine the effect of PF4 on corneal neovascularization involves the implantation of angiogenic stimuli into a surgically-created micropocket in the cornea of animals. The assay is often conducted using rats or rabbits. The angiogenic stimuli induces a vigorous neovascular response which can be easily detected in this normally avascular tissue. PF4 is incorporated, along with the angiogenic stimuli, into the implant pellet in order to assess the effect of PF4 on the neovascularization process. This assay demonstrates the ability of PF4 to effectively inhibit corneal neovascularization.

The aberrant development of retinal capillary networks is a cause of significant vision loss in several disease conditions such as proliferative diabetic retinopathy, disciform macular degeneration, and retinopathy of prematurity (or retrolental fibroplasia). While the precise etiologic events or agents causing these conditions is unknown, all three are associated with retinal ischemic damage following a transient decrease of the oxygen supply in the tissue. The current understanding of these conditions suggests that the common event of transient altered oxygen levels induces tissue damage, the release of mitogenic cytokines, and vigorous vascular endothelial cell migration, proliferation, and assembly. The conditions associated with retinal neovascularization can be simulated in neonatal rats. The relatively avascular retina of the full term newborn rat is similar in morphology to that of the minimally vascularized retina of a premature human baby. Under conditions of hyperbaric oxygen followed by normal air, a pathology is induced in both species' retinas, which is remarkably similar as assessed morphologically and histologically. The retinal pathology associated with the adult diseases mentioned above presents a similar morphological pattern which is believed to be caused by the same type of transient ischemic event. Thus, the neonatal rat model is a major step forward in the identification of broadly active antiproliferative drugs for treatment of neovascular retinopathy. Therefore, the positive results reported here establish that PF4 effectively

inhibits retinal neovascularization. Furthermore, the combined results showing the advantageous activity of PF4 in both the cornea model and the retina model establish that this protein is effective across a range of ocular diseases when those diseases involve neovascularization.

Data has also been obtained establishing the utility of PF4 in treatment of Kaposi's sarcoma. This human clinical data was obtained from twelve patients with Kaposi's sarcoma (KS) who were treated with PF4 doses ranging from 0.1 to 2.5 mg/lesion daily for 7 days, then three times a week for 3 additional weeks. Each subject had two randomly selected lesions chosen for study, and a prospective randomized blinded administration of study drug or control was performed. Each patient had one lesion injected with rPF4 and one lesion injected with saline. No serious life-threatening adverse events occurred; two patients died of other AIDS-related events. Mild to moderate erythema was observed at the injection site in approximately 20% of the patients. Of 12 patients injected, 6 patients demonstrated some anti-KS effects, and all 3 patients at the highest dose demonstrated anti-KS effects. This study demonstrates the safety and utility of rPF4.

The results of our study with people suffering with Kaposi's sarcoma further highlights one of the critical advantageous features of PF4—its lack of *in vivo* toxicity at dosages useful for ameliorating disease. This characteristic of PF4 makes PF4 particularly useful as a cancer treatment. The fortuitous biodistribution profile observed for PF4 further accentuates the surprising low toxicity of PF4 and is an important characteristic of this compound enabling its systemic use.

The findings reported herein establish that PF4, as an inhibitor of angiogenesis, has clinical utility in the management and detection of widely disseminated sites of angiogenesis and cellular proliferation. For example, progressive growth of tumors requires new blood vessel formation which, if inhibited, may not only restrict tumor growth, but stimulate regression of existing vessels, as well as enhance other responses (such as immune responses) to malignant invasion. As described below, the subject invention is particularly advantageous when the angiostatic activity of PF4 is combined in a synergistic way with toxins which destroy existing tumor cells.

PF4 as a targeting agent. The applicants' discovery that PF4 preferentially binds to proliferating endothelial cells is the basis for the use of PF4 as a targeting agent. This embodiment of the invention is highly advantageous because it enables the skilled artisan to accurately and predictably direct any one of a variety of biological activities to sites of endothelial cell proliferation located throughout the body. Specifically, when PF4 is used as a targeting molecule, various pharmaceutical, cytotoxic, or diagnostic agents can be covalently or non-covalently coupled to the PF4 to form a conjugate. Examples of such useful agents include: radioactive compounds (e.g., $^{125}I$, $^{131}I$); agents which bind DNA, such as alkylating agents or various antibiotics (daunomycin, adriamycin, chlorambucil); anti-metabolites (e.g., methotrexate); inhibitors of protein synthesis (e.g., diphtheria toxin and toxin plant proteins); and cytokines (e.g., tumor necrosis factor, interferon, and interleukin-2).

PF4 can be particularly advantageous as a drug delivery vehicle because of its heparin-binding characteristics. Pre-incubation with heparin before injection will prevent PF4 from binding to the vascular wall. There is evidence that the endothelium in different vascular beds is coated by unique species of heparin sulfate proteoglycans. Thus, by incubating PF4 with a mixture of unique species of heparin sulfate, PF4 may be directed predominantly to specific locations.

In diagnostic applications, PF4 can be conjugated to a label which generates a detectable signal. The conjugate is administered to an animal suspected of having a tumor, other pathological angiogenesis, or normal angiogenesis which is desired to be detected. After sufficient time to allow the detectably labeled PF4 to localize at the angiogenesis site, the signal generated by the label can then be detected by an appropriate detection device. The detected signal can then be converted to an image of the site. This image makes it possible to localize the angiogenesis site *in vivo*. In the case of pathological angiogenesis, this data can be used to develop an appropriate therapeutic strategy or to monitor disease progression. Such procedures can also be used *in vitro* to, for example, assess the angiogenic activity of excised tissue.

In one embodiment of the subject invention, a PF4 conjugate can be used in either an *in vivo* or *in vitro* assay to assess whether a tumor has angiogenic activity characteristic of malignant tumors. For example, tumor tissue which has been removed from an animal can be contacted with a composition comprising PF4 bound

to a detectable label. After a period of time sufficient to allow the PF4 conjugate to bind to any proliferating cells, the tumor sample can be separated from the PF4-conjugate solution and the sample can then be analyzed to determine the extent to which PF4 bound to the tissue. A high incidence of PF4 binding is associated with active cellular proliferation characteristic of a malignant tumor. This procedure also could readily be utilized by a person skilled in the art to assess the malignancy of tumors *in vivo*.

The PF4 of the subject invention can also be used in a pregnancy test. Preferably, in this embodiment, the PF4 can be conjugated in a manner which reduces or eliminates angiostatic activity while maintaining the ability to bind to sites of endothelial cell proliferation. Alternatively, pregnancy could be avoided or halted by the use of a PF4 conjugate that inhibits angiogenesis.

The labels that are suitable for conjugation to PF4 for angiogenesis localization include, but are not limited to, radio labels (e.g., radioisotopes) (also referred to herein as radiation emitters), fluorescent labels, and biotin labels. Radioisotopes that are suitable for labeling PF4 include Iodine-131, Iodine-123, Iodine-125, Iodine-126, Iodine-133, Bromine-77, Indium-111, Indium-113m, Gallium-67, Gallium-68, Ruthenium-95, Ruthenium-97, Ruthenium-103, Ruthenium-105, Mercury-107, Mercury-203,Rhenium-99m,Rhenium-105,Rhenium-101,Tellurium-121m,Tellurium-99m, Tellurium-125m, Thulium-165, Thulium-167, Thulium-168, Technetium-99m, and Fluorine-18. The gamma-emitting Indium species and Technetium-99m are preferred isotopes because these isotopes are detectable with a gamma-camera and have favorable half lives for imaging *in vivo*.

Fluorescent compounds suitable for labeling PF4 include fluorescein sodium, fluorescein isothiocyanate, and Texas red sulfonyl chloride. Those skilled in the art will, with the benefit of the current application, be able to utilize other detectable compounds that are suitable for labeling PF4.

In one embodiment of the subject invention PF4 is used to direct a diagnostic agent for magnetic resonance imaging of tumors to sites of cellular proliferation or neovascularization. Specifically, chelators for indium or gadolinium such as diethylenetriaminepentaacetic acid may be conjugated according to the protocols provided herein. In another embodiment chlorin-e6 or related photoactivable

molecules may be conjugated to PF4 as a means of photodynamic therapy for surface accessible vascular diseases such as diabetic retinopathy. In yet another embodiment, neutron activatable molecules such as Boron-10 can also be conjugated to PF4 for Boron neutron capture therapy.

For selective chemotherapy, conjugation of well known chemotherapeutic drugs such as methotrexate, ricin A, or doxorubicin to PF4 can be used to enhance the potency and reduce the toxic side effects of these drugs. PF4 acts as a targeting agent for tumor neovasculature sites and is able to more efficiently accumulate the conjugated drugs at those sites thereby reducing the tumor as well as inhibiting angiogenesis.

In a further embodiment, attachment of a DNA fragment via, for example, an acid hydrolyzable bond to PF4 can be used to facilitate efficient targeting of genes to proliferating endothelial cells. Upon endocytosis, the DNA fragment is captured into the endosomes and eventually released into the cell where it expresses the desired gene product.

In another embodiment of the invention, PF4 can be used in angiography. For example, ophthalmologists currently carry out fluorescein angiograms by giving a single intravenous injection of fluorescein and then taking rapid photographs of the eye using blue light or cobalt light to fluoresce the blood vessels in the retina. Neovascularization is detected by underlying leakage of fluorescein from the new vessels which leave a yellow perivascular stain. This is an indirect way of detecting neovascularization. Unfortunately, this procedure is subject to misinterpretation of the results because there are many other causes of leakage of fluorescein that are not caused by neovascularization.

The use of PF4 in angiography is advantageous because the fluorescein does not escape from the vessels and is visible for at least one to two hours, thus providing the ophthalmologist with a "standing angiography." In this way, the ophthalmologist has time to look directly at the eye and examine it without having to wait for films to be developed. Films can also be taken to provide a permanent record.

Fluorescein-labeled PF4 can also be used to augment laser therapy. Conventional laser therapy for diabetic neovascularization aims at the destruction of the retina which produces angiogenic peptides. By keeping fluorescein within the

vessel wall, the ophthalmologist can laser new capillaries directly, thereby sparing the retina. Conventional fluorescein disappears from circulation so rapidly that the vessels cannot be stained. Therefore, the use of PF4 labeled with a fluorescent marker can improve laser therapy in ophthalmology. Methods of forming these conjugates are known in the art and are discussed in detail herein. These conjugates can then be administered in an appropriate carrier as described below.

Mutants, variants, and fragments of PF4. The subject invention embraces the specific amino acid sequences and other compositions which are specifically exemplified herein. The subject invention further embraces analogs and variants of these sequences, as well as fragments of the sequences, and analogs and mutants of the fragments. As used in this application, the term "analog" refers to compounds which are substantially the same as another compound but which may have been modified by, for example, adding side groups. "Mutants" and "variants" as referred to in this application refer to amino acid sequences which are substantially the same as another sequence but which have amino acid substitutions, additions, or deletions at certain locations within the amino acid sequence or at the ends of the sequence. "Fragments" refer to portions of a longer amino acid sequence. These analogs, variants, and fragments are embraced within the subject invention so long as the analog, fragment, or variant retains substantially the same relevant biological activity as the originally exemplified compound. For example, it is well within the skill of a person trained in this art to make conservative amino acid substitutions. These substitutions are discussed in more detail below. To the extent that these substitutions do not substantially alter the relevant biological activity, then the resulting compounds fall within the scope of the subject invention. The term "relevant biological activity" refers to the activity of interest for a particular application of a compound. For example, several uses of PF4 are discussed below. These uses include inhibition of angiogenesis and endothelial cell proliferation and binding to proliferating endothelial cells. When PF4 is being used in these ways then "variants" would refer to compounds where PF4 has been modified (by a conservative amino acid substitution, for example) without, for example, substantially altering the compound's ability to inhibit angiogenesis or endothelial cell proliferation or bind to proliferating endothelial

cells. Conservative amino acid substitutions are only one example of the type of modifications which are within the scope of the subject matter of this invention.

As described herein, full length recombinant PF4 inhibits growth factor-dependent human endothelial cell proliferation *in vitro*. Significantly, it has also been determined that the angiogenesis-inhibiting activity of PF4 is retained by synthetic peptides corresponding to sequences of PF4 as small as 10 amino acids in length. In particular, a synthetic peptide of 13 amino acids corresponding to the carboxy terminus of PF4 (C-13) has displayed potent angiostatic activity. A peptide of 41 amino acids corresponding to the carboxy terminus of PF4 (C-41) has also shown angiostatic activity.

The activity of the C-13 peptide is especially surprising in light of its inability to affect the anticoagulant activity of heparin. The use of the C-13 peptide offers several advantages over whole PF4 such as reduced dosage (weight basis), reduced likelihood of antigenicity, and greater likelihood of effectiveness in novel dosage forms.

The C-13 peptide of PF4 also retains the ability to prevent Con-A induced immunosuppression in mice, an activity which is unaffected by heparin and probably independent of the ability of the peptide to inhibit angiogenesis.

It is well understood that angiogenesis is required for solid tumors to grow beyond a few cubic millimeters. Thus for the treatment of solid tumors, use of PF4, or modifications thereof, to inhibit angiogenesis presents a novel and highly advantageous means of therapy. The fact that the C-13 peptide inhibits angiogenesis without affecting the anticoagulant activity of heparin demonstrates that this small peptide also has the benefit of not interfering with concurrent anticoagulant therapy. Additionally, small peptides are generally less antigenic than larger proteins, and, thus, the PF4 fragments of the subject invention can be used advantageously for oral and transdermal administration. These types of delivery are particularly useful in the treatment of gastrointestinal capillary proliferation and skin lesions (e.g., Kaposi's sarcoma), respectively. Intralesional, as well as systemic, administration of PF4 fragments are also appropriate for treatment of these conditions. Topical or aerosol administration of PF4 fragments is appropriate for skin or pulmonary lesions, respectively (e.g., Kaposi's sarcoma and lung cancer).

Variants of PF4 which exhibit enhanced ability to inhibit angiogenesis have been synthesized. One such variant, known as rPF4-241, was created by cassette mutagenesis of a synthetic PF4 gene whereby four lysine residues of the carboxy terminus of PF4 were converted to two Gln-Glu couplets in order to eliminate heparin binding activity while retaining the α-helical secondary structure.

Synthesis of PF4 conjugates. One important aspect of the subject invention is the discovery of a Preferred Conjugation Site (PCS) on the PF4 molecule. By attaching a molecule with a desired activity to PF4 at the PCS, it is possible to produce a conjugate which retains PF4 biological activity, yet also has the desired activity attributable to the molecule which is conjugated to the PF4. In a preferred embodiment of the subject invention, PF4 is conjugated to other molecules through chemical processes which modify a free carboxyl group of an amino acid in the PF4 molecule. This modification has been found to be most preferable when the conjugation occurs near the N-terminus of the PF4 molecule. Thus, a further aspect of the preferred mode of the subject invention is the discovery that conjugation involving modification of carboxyl-containing amino acid residues located at, or near, the N-terminus of the PF4 molecule can be particularly effective in the production of a biologically active conjugate. Particularly attractive targets for modification are amino acids possessing side chains containing free carboxyl groups such as glutamic acid-1, glutamic acid-3, and glutamic acid-4. The free carboxyl group of the aspartic acid residues can also be modified. Aspartic acid residues 5 and 7 are preferred sites for modification. It was found that conjugation to PF4 at one of the first 14 amino acids of the N-terminus of PF4 advantageously results in the production of a biologically active conjugate.

Activation of carboxyl residues is the underlying principle of preferred coupling methods used according to the subject invention. C-activation can be achieved by attaching an electron withdrawing atom (e.g., chlorine) or group (e.g., azide) which renders the carbon atom of the carboxyl group sufficiently electrophilic to facilitate nucleophilic attack by nucleophilic reagents such as simple or complex molecules or polymers containing α-amino or hydrazide groups. O-activation is an alternative method to generate an activated site at the carboxyl residue. Conversion

of the carboxyl-component into a highly reactive acid anhydride can be achieved using reagents such as acid chlorides or alkyl carbonates. Such O-activation can also be in the form of active esters which are generally more stable in the reaction media such as the various halogen or nitro substituted phenolates, cyanomethyl esters, substituted hydroxylamines, substituted carbodiimides, and substituted azoles such as benzotriazoles.

In a preferred embodiment of the subject invention, a carbodiimide or bis-carbodiimide is used as an activating agent to facilitate the conjugation of desired moieties to the free carboxyl groups of PF4. Various carbodiimides and bis-carbodiimides which can be used according to the subject invention are known to those skilled in the art. These reagents serve to activate the free carboxyl groups of amino acid residues to facilitate conjugation with a desired entity such as a label or toxin. In a preferred embodiment, the entity to which the PF4 is conjugated will have an activated nucleophile. Such activated nucleophiles include, but are not limited to, amines, hydrazides, and alcohols. Specific examples of such modifications include conjugations with aminoacetamidomethyl fluorescein, PEG-hydrazide, or biotinamidocaproyl hydrazide. These modifications, and the biological activity of the modified products are described in more detail in the Examples which follow.

Although modification of carboxyl groups may be preferred, as described above, for certain embodiments, modification of amino groups can also be used. One specific chemical modification of PF4 according to the subject invention which results in advantageous biological properties involves modification of the free amino groups of rPF4 with fluorescein-isothiocyanate (FITC). The resulting adduct, FrPF4, surprisingly, retains the ability to inhibit angiogenesis as tested in the human umbilical vein endothelial cell (HUVEC) proliferation assay. Thus, angiostatic activity is also found in PF4 fragments and mutants which have been modified with the bulky and hydrophobic fluorescein moiety. In addition to their biological activity, the FITC-labeled PF4 sequences are useful for visual detection of PF4 molecules. Furthermore, the ability to modify PF4 and its fragments with large moieties without loss of the relevant biological activity provides a basis for conjugating PF4, its fragments, mutants, or derivatives with toxins, monoclonal antibodies, polyclonal antibodies, fluorophores, cell receptor molecules, non-proteinaceous biological effector molecules,

chelators, linkers, carrier proteins, polysaccharides, polyamino acids, and other large entities. The linkers used according to the subject invention can preferably comprise hydrolyzable bonds. These bonds may be acid, enzymatic, or spontaneously hydrolyzable. The conjugation may occur through, for example, modification of functional groups on the protein, such as free amino, carboxyl, sulfhydryl, or guanadinium (arginine) groups of PF4. Also, conjugation to aromatic groups such as possessed by histidine and tyrosine can be done. A further option is the modification of hydroxy groups. The modification of these moieties necessary to effect the desired conjugation can be carried out by chemical procedures described herein as well as those which are well known to those skilled in this art. See, for example, Ming Lu *et al.* (1992) *J. Immunol. Meth.* 156:85-99.

Administration of PF4 conjugates. The compounds of the subject invention can be combined with a suitable pharmaceutical carrier. For example, these compounds can be formulated in physiologically acceptable carriers, such as phosphate buffered saline, acetate buffered saline, distilled water, excipients, or the like, or may be administered neat.

In accordance with the teachings of the subject invention, PF4 can be used as an active agent for treatment of inappropriate angiogenesis, or PF4 can be used as a targeting agent for selectively delivering other agents to sites of inappropriate angiogenesis. When PF4 is used as the active ingredient, a physiologically-acceptable solution of PF4 is administered into the afflicted animal by any appropriate means including, but not limited to, injection into a blood vessel; direct application to a surgical site using a biocompatible gel, film, or sponge; intraperitoneal or subcutaneous injection; topical administration; and pulmonary administration.

The compositions used in these therapies may also be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspension, liposomes, suppositories, injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. The compositions also preferably include conventional pharmaceutically acceptable carriers and adjuvants which are known

those of skill in the art. Preferably, the compositions of the invention are in the form of a unit dose and will usually be administered to the patient one or more times a day.

PF4 conjugates may be administered to the patient in any pharmaceutically acceptable dosage form, including intravenous, intramuscular, intralesional, or subcutaneous injection. It should, of course, be understood that the compositions and methods of this invention may be used in combination with other therapies.

Once improvement of the patient's condition has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, may be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment can cease. Patients may, however, require intermittent treatment on a long-term basis upon any recurrence of disease symptoms.

If PF4 is administered intralesionally, it can be applied such that the dosage is between about 1.0 µg/lesion and about 10 mg/lesion. For systemic administration, the dosage of PF4 can be between about 100 µg/kg of body weight and about 500 mg/kg of body weight. Similar and higher dosages can be used for the administration of PF4 conjugates and peptide fragments. For example, dosages of PF4 fragments may be twice that of PF4 or higher.

Ligands conjugated to PF4 can also be utilized according to the subject invention. In one embodiment of the subject invention, PF4 can be bound to biotin using procedures known to those skilled in the art and described herein. The PF4-biotin complex can then be administered to an animal concurrently with, or followed by, an avidin complex. The avidin complex can comprise avidin and a second component bound to the avidin. For diagnostic applications the second component can be a detectable label such as a radiolabel. For therapeutic applications the second component can be any of the various toxins, chemotherapeutic agents and other therapeutic molecules described herein or otherwise known to those skilled in the art. In this embodiment of the subject invention, PF4 directs the PF4-biotin conjugate to a site of angiogenesis or cellular proliferation. The avidin conjugate then binds to the biotin thereby bringing the second component of the avidin conjugate to the site of angiogenesis or cellular proliferation. The second component of the avidin conjugate can then either be detected in a diagnostic procedure, or exert its therapeutic effect.

In the therapeutic embodiment of this example, a synergistic activity can result from the combined actions of the PF4 which inhibits angiogenesis and cellular proliferation together with the toxin effect of the toxin which destroys existing cells involved in inappropriate angiogenesis.

One important aspect of the subject invention concerns the discovery that PF4 can be delivered systemically without unwanted side effects or the loss of desirable biological activity. The efficacy of systemically-administered PF4 has been demonstrated using several models. These models, which are described in detail in the Examples which follow, provide valuable insight into the bio-distribution patterns of systemically-administered PF4 and PF4 conjugates.

The *in vivo* inhibition of metastatic tumor growth following systemic administration of PF4 has been demonstrated. The data specifically demonstrate activity against liver and lung metastases and further demonstrate that the activity is not dependent upon the mode of systemic administration. Intravenous, subcutaneous, and intraperitoneal administration have all been used with great success. These results show that PF4 is efficiently transported systemically to the target site.

The results obtained using animal models are particularly reliable in view of the biodistribution data which has also been obtained. This biodistribution data provides further evidence of the utility of PF4 for treating disseminated sites of neovascularization. Also, toxicity data, including data obtained from human clinical trials, establish the safety of PF4.

As described in the Examples which follow, the applicants have conducted experiments which enable visual monitoring of the distribution of PF4 which has been administered systemically. In one assay, the movement of PF4 in hamster cheeks is monitored after the systemic administration of the PF4. In the other assay, the movement of PF4 is monitored near a site of induced angiogenesis. The PF4 in this assay was also administered systemically. In each assay, the PF4 is conjugated to FITC, which facilitates visual monitoring of the PF4 distribution over a period of time. With each of these two methods PF4 was observed to preferentially accumulate at sites of active angiogenesis. These results demonstrate the ability of PF4 to advantageously exert its own angiostatic activity at sites of neovascularization.

Moreover, the binding of PF4 conjugates at sites of neovascularization provides an effective means of targeting therapeutic and diagnostic agents to these sites.

## Materials and Methods

Chicken chorioallantoic membrane (CAM) assay. Fertile eggs were incubated in a stationary position for 3 days at 37°C and 70-80% relative humidity. During this time, the embryo rose to the upper surface of the egg contents. At the beginning of the 4th day, the eggs were cracked without inversion and carefully deposited into sterile plastic petri dishes such that the embryo remained on the upper surface. The shell-free eggs were incubated for an additional 72 hours at 37°C, under an atmosphere containing 2.5-3.5% $CO_2$ after which the growing embryos developed a recognizable CAM. Discs, made by mixing test samples with 1% (w/v) methylcellulose were dried and placed on the CAM between major veins and approximately 0.5 cm from the embryo. Following another 48 hour incubation at 37°C (2.5-3.5% $CO_2$), the samples were scored for their ability to inhibit angiogenesis. Inhibition appears as an avascular zone surrounding the implant and can often include elbows formed by veins avoiding the disc and a reduced number of capillaries in the region of the implant.

Endothelial cell proliferation assay. Human umbilical vein endothelial cells were cultured in Medium 199 (Gibco) containing 10% (v/v) fetal bovine serum (FBS), 150 mcg/ml endothelial cell growth supplement (ECGS) and 5 units/ml heparin at 37°C and 4-5% $CO_2$. Every 3-4 days, the cultures were harvested by trypsin treatment, diluted, replated, and grown to confluence. Prior to the start of an experiment, the cells were centrifuged and resuspended in heparin-free media and incubated with the test substance for 3 days under standard culture conditions. At the end of the incubation period, the cells were harvested and counted. Statistical significance between means was determined by a standard Student t-test for unpaired data.

rPF4 production. Recombinant PF4 was produced in *E. coli* as an N-terminal fusion protein containing a methionine immediately preceding the PF4 sequence. The insoluble fusion protein was cleaved with cyanogen bromide treatment and purified by heparin agarose affinity chromatography. The isolated protein was buffer

exchanged into 20 mM sodium acetate, pH 4.0, and either frozen or lyophilized for storage.

Production of peptides. Peptides were prepared by standard solid phase synthesis procedures, cleaved from the solid support and deblocked, and purified by reverse phase HPLC.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 — CAM Assay

Chicken eggs, prepared as described above, were treated with discs containing several concentrations of recombinant PF4 or peptides derived from the sequence of PF4. rPF4 and C-terminal peptides as small as 13 amino acids inhibited angiogenesis on the CAM (Figure 1). In each case, the inhibition was dose-dependent and the response approximately equivalent (molar basis) for the inhibitors containing the C-terminal region of PF4. An N-terminal peptide of PF4 (N-29) did not inhibit angiogenesis even at the highest concentration tested, suggesting that all of the anti-angiogenic activity of PF4 is associated with the C-terminal portion of the molecule. Since the C-terminus of PF4 is rich in lysine, polylysine was tested in this assay system and found not to cause inhibition at 6.5 nmol dosages.

Example 2 — Heparin Binding of PF4 Fragments

The lysine rich region of PF4 (residues 61-66) is also the domain associated with the binding of heparin by PF4. Heparin is known to play a role in modulating angiogenesis, which can also be affected by protamine, another well characterized heparin-binding protein. To assess the ability of PF4-based synthetic peptides to bind heparin, we assayed the activity of coagulation-cascade enzymes which are inhibited by heparin. Protamine and platelet factor 4 are able to prevent the heparin inhibition of thrombin and Factor Xa at approximately equimolar concentrations. The 41 amino acid C-terminal peptide of PF4 (C-41) prevented heparin inhibition less effectively,

but the C-13 peptide was unable to prevent the inhibition of thrombin even at concentrations ten times that of an effective level of rPF4. This unexpected finding suggests that the C-13 peptide inhibits angiogenesis by some method other than heparin binding.

Example 3 — Inhibition by PF4 of Growth Factor Stimulated Cellular Proliferation

Endothelial cell division and growth is tightly controlled and strictly dependent on the presence of growth factors. We evaluated the ability of rPF4 and related peptides to inhibit growth factor-stimulated human endothelial cell proliferation *in vitro*. rPF4 significantly inhibited endothelial cell growth in a dose-dependent fashion at a concentration as low as 10 mcg/ml. Inhibition was complete at 25 mcg/ml in the heparin-deficient medium employed here.

Example 4 — Effect of Heparin Binding on Antiproliferative Activities of PF4

To assess the importance of the heparin binding activity of PF4 in the inhibition of endothelial cell proliferation, cells were incubated in media containing or lacking 5 units/ml heparin. The presence of heparin stimulated proliferation of these cells during the three day incubation of this experiment. rPF4 significantly inhibited both control (100%) and heparin stimulated (45%) endothelial cell growth (Table 1).

Table 1. Attenuation of rPF4 inhibition of endothelial cell growth by heparin.

| Addition | rPF4 | | %Inhibition[a] |
|---|---|---|---|
| | -- | 50mcg/ml | |
| -- | 14.4 ± 2.5 | [b]6.0 ± 0.6 | ≈100 |
| 5 u/ml heparin | 18.9 ± 1.2 | [b]14.0 ± 0.4 | 45 |

[a]Based on seeding of 8 x $10^4$ cells/well
[b]Significantly different from appropriate control ($p < 0.005$)

Example 5 — Construction of rPF4-241

Cassette mutagenesis of a synthetic PF4 gene was used to convert the four lysine residues at the carboxy terminus of PF4 to two Gln-Glu couplets (see Figure 2). This construction apparently retains the α-helical secondary structure (Figure 3) for this region of the molecule with the concurrent loss of heparin binding activity.

The gene for rPF4-241 was expressed as a fusion protein in *E. coli* with the same N-terminal amino acid sequences as with the parent rPF4 molecule. The protein was cleaved from the *E. coli* fusion peptide by CNBr and formic acid and purified to homogeneity by DEAE-sepharose chromatography. The protein was reactive with polyclonal antibodies to PF4 and was determined to possess the appropriate modifications by amino acid analysis. Significantly, the purified mutant protein lacked heparin-binding activity in the Factor Xa inhibition assay.

The substitutions described here can be made with the peptide fragments as well as with the full length PF4 molecule. For example, C-13-241 has the following sequence:

Pro-Leu-Tyr-Gln-Glu-Ile-Ile-Gln-Glu-Leu-Leu-Glu-Ser (SEQ ID NO. 1).

Example 6 — Inhibition of Angiogenesis by rPF4-241

Purified rPF4-241 was dried in methylcellulose discs and tested for its ability to inhibit capillary growth in the chicken chorioallantoic membrane (CAM) assay. Even at the lowest concentrations tested (1.25 nmol/disc) rPF4-241 extensively inhibited angiogenesis in the CAM system (Figure 4). This inhibition was even more effective than that caused by equal concentrations of native rPF4 as suggested by larger avascular zones on the membrane. The inhibitory effect of rPF4-241 was not reversed by heparin.

Example 7 — Inhibition of Human Endothelial Cell Proliferation by rPF4-241

At concentrations where native rPF4 completely inhibits endothelial cell proliferation, mutant rPF4-241 was at least as effective in inhibiting cell growth (Figure 5). Further tests suggest that rPF4-241 was inhibitory at concentrations as low as 0.5 mcg/mL.

In a test of inhibition of human umbilical vein endothelial cell proliferation by native rPF4 and mutant rPF4-241, the rPF4-241 was shown to be more effective than the native rPF4 at inhibiting the proliferation of these cells. The results of this test are shown in Figure 6.

These results are remarkable in that previous theories of PF4 inhibition of angiogenesis assumed that the PF4 effects were due to heparin binding. We have designed a protein, retaining most of the structural features of native PF4 but lacking detectable heparin binding activity, which is apparently more active than native PF4 in inhibiting angiogenesis *in vivo* and endothelial cell proliferation *in vitro*. Additionally, the mutant we have designed would not be expected to interfere with heparin anticoagulant therapy.

Example 8 — Inhibition of *In Vivo* Tumor Growth by rPF4 and its Variants

Normal C57BL/6J female mice (6-8 weeks old) were inoculated subcutaneously with $5 \times 10^5$ log phase cells of a B16-F10 melanoma tumor line. This protocol led to progressive tumor growth resulting in large (300 $mm^3$) necrotic tumors after approximately 10 days, followed by death of untreated animals usually within three weeks of tumor inoculation.

In an experiment to test the efficacy of rPF4 in preventing *in vivo* tumor growth and angiogenesis, tumor bearing animals were divided into two groups. One group was injected with 50 µg rPF4 (native sequence) in 100 µl of 50 mM sodium phosphate, pH 6.5, 50 mM sodium chloride directly into the nascent tumor, daily, beginning one day after tumor inoculation. A control group was treated identically with carrier buffer lacking rPF4. Tumor volume was measured at regular intervals with digital calipers by laboratory personnel uninformed of the specific treatment received by each subject animal.

Within seven days of tumor inoculation, control animals possessed obvious three dimensional tumors, while rPF4-treated animals were essentially tumor-free (Figure 7). Continued treatment with rPF4 completely suppressed tumor growth under these conditions where control animal tumors became necrotic and large as seen previously with untreated mice. The same effect was observed when rPF4-241 was used as the inhibitory agent.

The finding that rPF4 inhibition of *in vivo* tumor growth was apparent within three days of the initial inoculation (of rPF4) indicates that rPF4 acts to modulate tumor growth by local mechanisms rather than by immunomodulation which would require a longer time course. Additionally, rPF4 did not directly inhibit tumor cell growth *in vitro*. It appears, therefore, that rPF4 was modulating the host's angiogenic response to the growing tumor.

It has been shown that proteins of identified structure and function may be constructed by changing the amino acid sequence if such changes do not alter the protein secondary structure (Kaiser, E.T., F.J. Kezdy [1984] *Science* 223:249-255). Thus, the subject invention includes mutants (or variants) of the amino acid sequences exemplified herein which do not alter the protein secondary structure, or if the structure is altered, the biological activity is retained.

We have conducted extensive research to determine what mutations can be made to the carboxy terminus of the PF4 sequence and still retain biological activity. Table 2 provides a listing of examples of several mutant sequences and their biological activity.

**Table 2.** Mutant sequences of rPF4 and their biological activity

| Designation | Sequence | CAM Activity |
|---|---|---|
| rPF4-211 (SEQ ID NO. 2) | [PF4 AA 1-57] - Pro Leu Tyr Lys Lys Ile Ile Lys Lys Leu Leu Glu Ser | pos. |
| rPF4 231 (SEQ ID NO. 3) | [PF4 AA 1-57] - Pro Leu Tyr | neg. |
| rPF4 241 (SEQ ID NO. 4) | [PF4 AA 1-57] - Pro Leu Tyr Gln Glu Ile Ile Gln Glu Leu Leu Glu Ser | pos. |
| rPF4 302 (SEQ ID NO. 5) | [PF4 AA 1-57] - Pro Leu Tyr Gln Gln Ile Ile Gln Gln Leu Leu Glu Ser | neg. |
| rPF4 303 (SEQ ID NO. 6) | [PF4 AA 1-57] - Pro Leu Tyr Lys Lys Gln Glu Lys Lys Gln Glu Glu Ser | pos. |
| rPF4 307 (SEQ ID NO. 7) | [PF4 AA 1-57] - Pro Leu Tyr Gln Ile Glu Ile Gln Leu Glu Leu Glu Ser | pos. |
| rPF4 308 (SEQ ID NO. 8) | [PF4 AA 1-57] - Pro Leu Tyr Asn Asp Ile Ile Asn Asp Leu Leu Glu Ser | pos. |
| rPF4 315 (SEQ ID NO. 9) | [PF4 AA 1-57] - Pro Leu Tyr Gly Glu Ile Ile Gly Glu Leu Leu Glu Ser | pos. |

Construction of the various mutants was accomplished via cassette mutagenesis of a synthetic gene as described in Example 5 above. This process is well known to any person of ordinary skill in the art. The results of the research demonstrate that a high percentage of the mutants retained angiostatic activity in the CAM assay. Although not every mutant retains this activity, from the teachings herein it is well within the skill of a person trained in this art to make desired mutations and determine whether such activity has been retained.

In particular it should be understood that conservative substitutions of amino acids may be made. For example, amino acids may be placed in the following classes: basic, hydrophobic, acidic, polar, and amide. Substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution does not materially alter the biological activity of the compound. Table 3 provides a listing of examples of amino acids belonging to each class.

**Table 3.**

| Class of Amino Acid | Example of Amino Acids |
|---|---|
| Nonpolar | Ala, Val, Leu, Ile, Pro, Met, Phe, Trp |
| Polar | Gly, Ser, Thr, Cys, Tyr, Asn, Gln |
| Negatively charged | Asp, Glu |
| Positively Charged | Lys, Arg, His |

In some instances, non-conservative substitutions can also be made. For example, lysine may be substituted for with any of the following amino acids: Glu, Gln, Asp, Asn, Met, Ala, Leu, and Ile. The critical factor is that these substitutions

must not significantly detract from the biological activity of the rPF4 or the rPF4 fragment.

The following sequence helps provide some additional guidance to a person skilled in this art in making various substitutions. It should be noted that this sequence is meant to be illustrative and not exhaustive, and that there may be other substitutions which eliminate heparin binding and retain angiostatic activity and, thus, are within the scope of the subject invention.

**A- Pro Leu Tyr $Xaa_{10}$ $Xaa_9$ $Xaa_8$ $Xaa_7$ $Xaa_6$ $Xaa_5$ $Xaa_4$ $Xaa_3$ Glu Ser - COOH**

(SEQ ID NO. 10)

wherein **A** represents all or part of the polypeptide sequence consisting of residues 1 through 57 of PF4; **A** may be or may not be present;

wherein $Xaa_{10}$ is Lys, Gly, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

$Xaa_9$ is Lys, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

$Xaa_8$ is Glu, Gln, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr;

$Xaa_7$ is Glu, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr;

$Xaa_6$ is Lys, Gly, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

$Xaa_5$ is Lys, Glu, Gln, Asp, Asn, Met, Ala, Leu, or Ile;

$Xaa_4$ is Lys, Glu, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr; and

$Xaa_3$ is Gln, Met, Ala, Leu, Ile, Val, Pro, Phe, Trp, or Tyr.

wherein, most preferably

$Xaa_8$ is Ile, Glu, or Gln;

$Xaa_7$ is Ile or Glu;

$Xaa_6$ is Leu, Gln, or Glu; and

$Xaa_5$ is Leu or Glu.

The amino terminus of the proteins of the subject invention can also be modified in a variety of ways while retaining the biological activity fundamental to the subject invention. Most notably, the length of the amino terminus can be modified while retaining angiostatic or endothelial cell inhibitory activity. Our reference here to the "amino terminus" refers to amino acids 1-60 from the amino terminal of PF4 or its variants. As we have shown herein, up to 57 of these amino acids can be removed with a retention of angiostatic activity. The remaining peptide is the

biologically active C-13 peptide. We have also shown that the C-41 peptide has biological activity. Thus, various active fragments of PF4 can be readily produced and used according to the subject invention. It would also be readily apparent to the skilled artisan that other modifications of the amino terminus can be made. For example, additional peptides or proteins can be added to that terminus, and various standard chemical derivatives can be made. Such modifications are within the scope of the subject invention so long as biological activity is retained.

It should be emphasized that the critical feature of the subject invention is the provision of polypeptides and polypeptide conjugates which either have angiostatic activity or antiproliferative activity, or are able to direct a second entity to sites of angiogenesis and neovascularization. As used herein, "angiostatic activity" refers to a level of angiostatic activity which is characteristic of PF4. This level of angiostatic activity is, for example, at least about 75% (and preferably, greater than about 90%) of the angiostatic activity exhibited by PF4 as measured, for example, in the CAM assay. Methods for determining angiostatic activity are described herein and are well known and readily performed by those skilled in this art. As used herein, the term "antiproliferative activity" refers to a level of antiproliferative activity which is characteristic of PF4. This level of antiproliferative activity is at least, for example, about 75% (and preferably, greater than about 90%) of the antiproliferative activity exhibited by PF4 as measured, for example, by the HUVEC assay. Methods for determining antiproliferative activity are described herein and are well known and readily performed by those skilled in this art. As used herein, the term "lack of heparin binding activity" refers to a relative lack of ability to bind heparin under normal physiological conditions compared to PF4. This lack of heparin binding activity is, for example, less than about 25% of PF4's heparin binding activity and, preferably, less than about 10% of PF4's heparin binding activity. The ability to bind heparin can be readily determined by various assays as described herein and as is known by those skilled in this art.

Example 9 — Modification of PF4 and rPF4-241 with Fluorescein-Isothiocyanate (FITC)

A. Purified rPF4 or rPF4-241 (5 mg in 50 mM $Na_2CO_3$, pH 9.3, 25 mM NaCl) was treated with 5 mg of fluorescein isothiocyanate in a volume of 5 ml to modify the free amino groups. After incubation for 3 hours at room temperature in the dark, the labeled protein (FrPF4 or FrPF4-241) was separated from unbound FITC by gel filtration and dialyzed into 50 mM acetic acid. A possible structure of the C-terminus of FrPF4 is shown in Figure 8.

B. In an alternative procedure rPF4 was batch bound to heparin-agarose at 1 mg protein/ml resin in 25 mM $NaH_2PO_4$, pH 8.0, 300 mM NaCl (Buffer F) for 24 hours at 4°C. 300 µg FITC/ml of heparin-agarose-protein complex was incubated for 18 hours in the dark at 4°C. The resin was then poured into an open column, washed with Buffer F and the protein was eluted with 25 mM $NaH_2PO_4$, pH 8.0, 2 M NaCl. The eluent was dialyzed against 40 mM acetic acid, pH 3.0 in a 3,000 MWCO dialysis membrane for 18 hours in the dark at room temperature. The dialyzed eluent was then loaded onto an S-Sepharose column equilibrated in 40 mM acetic acid, pH 3.0. The column was washed with 40 mM acetic acid, pH 3.0, 1 M NaCl, followed by an additional wash with 40 mM acetic acid, pH 3.0, 2 M NaCl. Protein was eluted with 50 mM Tris-HCl, pH 9.0, 2 M NaCl. The eluent was dialyzed against 0.1% TFA:H2O in a 3,000 MWCO dialysis membrane at room temperature in the dark. The protein was subsequently lyophilized and stored at -20°C in the dark. This procedure protects the heparin binding site before the amino groups are modified with the FITC.

Example 10 — Inhibition of Angiogenesis by Fluorescein-Isothiocyanate-Conjugated rPF4

FrPF4, produced as described in Example 9A, was tested for activity in the CAM assay as described above. Although FrPF4 lacked heparin binding activity, it retained full activity as an inhibitor of angiogenesis on the CAM. The results of these assays are shown in Table 4.

Table 4. Activity of FrPF4 in the CAM assay.

| Amount per disc (μg) | Inhibition (%) | |
|---|---|---|
| | rPF4 | FrPF4 |
| 0 | 0 | 0 |
| 10 | 22 | 17 |
| 25 | 33 | 33 |

Example 11 — Inhibition of Endothelial Cell Proliferation by FrPF4 and FrPF4-241

FrPF4 and FrPF4-241, produced as described in Example 9, were tested separately to determine their ability to inhibit endothelial cell proliferation. HUVE cells were tested for their sensitivity to FrPF4 as described above except that [$^3$H]-thymidine was added to the cultures 24 hours after the addition of FrPF4. The cultures were then incubated an additional 6 hours. Cells were harvested, washed, and radioactive thymidine incorporation into DNA was measured.

As shown in Figure 9, FITC-conjugated rPF4 was very effective, even at low dosages, in inhibiting DNA synthesis in human umbilical vein endothelial cells and therefore inhibiting cell proliferation. Similar results were obtained using FrPF4-241. In this case, the inhibition of HUVE cell proliferation with increasing concentrations of rPF4-241 was tested using the Endothelial Cell Proliferation Assay as described above. The results of experiments using FrPF4-241 are shown in Figure 10.

Example 12 — Inhibition of *In vivo* Tumor Growth by FITC-rPF4

FrPF4 was produced as described in Example 9. B-16 Melanoma tumors were grown in C57BL6/J mice as described previously. Treatment was begun 24 hours following implantation of tumor cells (Day 1) and consisted of 25 μg/day of FrPF4 in 100 μl of sodium acetate buffer, pH 4.0. Control mice were injected with 25

μg/day of FITC labeled cytochrome-C in the same buffer. A statistically significant suppression of tumor growth by FrPF4 was observed by Day 11 (Figure 11).

Example 13 — Inhibition of Liver Metastases with Systemic Administration of PF4

PF4 administered as multiple intraperitoneal injections has been found to inhibit the growth of liver metastases (murine M5076 reticulum cell sarcoma). This finding further establishes systemic antitumor activity of rPF4 and shows that the effect is neither tumor type nor organ site specific.

Inhibition of lung metastasis. Cells of a B16 murine melanoma cell line were injected intravenously into a tail vein of a mouse. Thirty seconds later, rPF4 (in saline or acetate buffer) was injected into the same mouse via a different tail vein. After 21 days, the tumor burden of the test and control animals was measured by counting, optically, metastases, and by weighing the lungs of the mice. Referring to Table 5, PF4 produced a dose-dependent effect, as measured by both parameters. Optimal results were observed when PF4 was administered systemically, when dosages were in excess of 5000 μg per kg of body weight; at these dosages, no observable toxicity or other adverse effects were noted.

**Table 5**

| Groups | Average* wt. (g) | Dose (mg/kg) | # of Metastases* (#/Mouse) | Lung* wt. (mg) |
|---|---|---|---|---|
| Control (Buffer only) | 17.7 | -0- | 136.2 | 443 |
| 0.375 mg rPF4 | 19.0 | 19.7 | 112.2 | 525 |
| 0.75 mg rPF4 | 18.7 | 40.1 | 66.0† | 404 |
| 1.5 mg rPF4 | 17.8 | 84.2 | 54.7† | 357 |

* Average of six mice per group.
† Statistically different from control group, $p<0.05$.

Example 14 — Inhibition of Retinal Angiogenesis with Systemic Administration of rPF4

Positive results have also been obtained demonstrating inhibition of retinal angiogenesis by rPF4 administered subcutaneously to neonatal rats. The anti-angiogenic activity of rPF4 in the rat model of ROP (retinopathy of prematurity, which is also known as retrolentro fibroplasia) was investigated. In these experiments, two litters of rat pups were exposed to 80% $O_2$ for 11 days, and then returned to air for 6 days. During $O_2$ exposure, animals were exposed daily to room air for 30 minutes, and then slowly returned to 80% over 3 hours. Every day from birth the animals were injected subcutaneously with rPF4 (50 mg/kg) or vehicle. One eye per animal was randomly chosen, the retina dissected leaving the ciliary body intact and the retinal vasculature displayed by ADPase histochemistry. Extraretinal neovascularization was scored and the area of avascular retina measured using Image software from NIH. The area of avascular retina was larger in the treated group than in the controls, although both groups exhibited extraretinal neovascularization. The mean area of avascular retina (AR) was 11.8 ± 6.8 $mm^2$ in the rPF4 animals (n=9) and 7.2 ± 3.9 $mm^2$ in controls (n=10). AR was >7 $mm^2$ in 78% of rPF4-treated animals vs. 40 in controls.

Example 15 — Inhibition of Corneal Neovascularization with PF4

Implantation of angiogenic or inflammatory stimuli into a surgically created micropocket in the cornea induces a vigorous neovascular response which can be easily detected in this normally avascular tissue. Encapsulation of rPF4 in the implant pellet effectively suppresses the angiogenic response to purified basic fibroblast growth factor in both rats and rabbits. See Table 6. Evaluation of the dose response relation of rPF4 suppression to bFGF stimulation showed rPF4 to be remarkably active in this assay system, with 20 ng of rPF4 capable of completely blocking the effect of 100 ng bFGF (Table 6) in both species. rPF4 alone induced neither an angiogenic nor an inflammatory response. An intact heparin binding domain was not required for this activity of rPF4 as demonstrated by the activity of the mutant, rPF4-241 (Table 6).

The absence of background vasculature permits the clear demonstration of distinct inhibitory effects of rPF4 and related molecules in this system and establishes their utility as *in vivo* angiogenesis inhibitors.

**Table 6.** Inhibition of bFGF Mediated Angiogenesis in Rabbit and Rat Corneas by rPF4 and Mutant rPF4

| | Angiogenic Activity<br>Proportion of Positive Responses (%) | | | | |
|---|---|---|---|---|---|
| Rabbits | bFGF (ng/cornea) | | | | |
| Material | -0- | 25 | 50 | 125 | 250 |
| PBS/HBSS | 0/4 (0) | 1/4 (25) | 3/4 (75) | 5/5 (100) | 5/5 (100) |
| rPF4 (ng) | | | | | |
| 5 | 0/5 (0) | 0/3 (0) | 0/3 (0) | 1/5 (20) | 1/5 (20) |
| 20 | 0/4 (0) | 0/4 (0) | 0/4 (0) | 0/3 (0) | 1/5 (20) |
| 100 | 0/3 (0) | 0/4 (0) | 0/4 (0) | 0/3 (0) | 0/4 (0) |
| Mutant rPF4 (ng) | | | | | |
| 5 | 0/3 (0) | 0/5 (0) | 0/5 (0) | 1/4 (25) | 1/4 (25) |
| 20 | 0/3 (0) | 0/5 (0) | 0/5 (0) | 1/4 (25) | 1/4 (25) |
| 100 | 0/4 (0) | 0/3 (0) | 0/3 (0) | 0/4 (0) | 0/5 (0) |
| Rats | bFGF (ng/cornea) | | | | |
| Material | -0- | 25 | 50 | 125 | 250 |
| PBS/HBSS | 0/5 (0) | 4/5 (80) | 5/5 (100) | 5/5 (100) | 5/5 (100) |
| rPF4 (ng) | | | | | |
| 5 | 0/5 (0) | 1/5 (20) | 0/4 (0) | 1/4 (25) | 4/5 (80) |
| 20 | 0/4 (0) | 0/4 (0) | 0/5 (0) | 0/5 (0) | 2/6 (33) |
| 100 | 1/5 (20) | 0/5 (0) | 0/5 (0) | 0/4 (0) | 1/7 (14) |
| Mutant rPF4 (ng) | | | | | |
| 5 | 0/4 (0) | 1/4 (25) | 1/5 (0) | 1/4 (25) | 2/5 (40) |
| 20 | 0/4 (0) | 0/5 (0) | 1/4 (25) | 1/5 (20) | 2/4 (50) |
| 100 | 0/5 (0) | 0/5 (0) | 0/5 (0) | 1/5 (20) | 1/5 (20) |

Example 16 — Treatment of Kaposi's Sarcoma with rPF4

The safety and efficacy of rPF4 when used to treat humans having lesions associated with Kaposi's sarcoma have been evaluated. To carry out this study, rPF4 was administered as a multiple dose (16 total) intralesional injection. Three lesions were monitored on each patient: one untreated, one treated with vehicle only, and one treated with rPF4 and the vehicle. The vehicle used for this study was a standard pharmaceutical carrier consisting of 10 mM sodium acetate and 150 mM sodium chloride at a pH of about 5.0. The two treated lesions were assigned and treated in a double blind fashion with neither the patient nor the physician aware of the specific agent in the coded syringes. The study utilized sets of three patients at each of four dose levels. The initial set of patients was treated with the lowest dose of rPF4. Additional sets of three patients were then treated at the higher dose levels. Each set of three patients completed the full course of treatment prior to entering three naive patients at the next higher dose level. A constant volume of 0.5 ml was used for injections at all four dose levels tested (0.1, 0.5, 1.5, 2.5 mg/lesion/treatment).

All patients completed the treatment protocol outlined above without reporting significant discomfort other than pain at the injection site, minor erythema in injected lesions, and occasional headaches which could not be definitively attributed to the treatments received. No evidence of hematological or serum chemistry abnormalities were seen in this study. Two patients could not be monitored as part of the long term follow-up due to progressive HIV associated disease.

In addition to the evaluation of toxicities, the characteristics of the treated (and untreated) Kaposi's sarcoma lesions were assessed in detail for signs of disease progression, stabilization, or regression. Lesion sizes (in two dimensions) were measured and documented on a regular schedule throughout the study. Lesion palpability and color were documented and assigned numerical scores according to a standard scale as well. Lesion biopsies were taken prior to and at the completion of therapy to evaluate subclinical evidence of biological effects of treatment.

Full assessment of the decoded quantitative data obtained in this study suggests that rPF4 inhibits lesion growth. Based on the clinical assessment of color, palpability, and other treated lesion characteristics, more of the rPF4-treated lesions were scored as showing signs of clinical response than were the placebo-treated

lesions. Two clinical investigators, each having no knowledge of treatment lesions as opposed to control lesions, recorded positive responses in 6 of 12 rPF4-treated lesions and 5 of 12 rPF4-treated lesions, respectively. These same investigators scored 3 of 12 and 2 of 12 placebo-treated lesions as positive responses, respectively. Thus, in each instance, at least twice as many lesions treated with rPF4 showed clinical response than did the controls. At the highest dose tested, one clinical investigator scored all three rPF4-treated lesions as showing evidence of a clinical response.

Example 17 — Treatment of Brain Tumors

Brain tumors can be treated with PF4 conjugates alone or in combination with radiation and/or chemotherapy, as follows:

A. Local treatment following surgery. Following surgical removal of a primary tumor lesion, residual tumor growth is prevented by direct treatment with PF4 conjugates. Treatment methods include direct injection of PF4 conjugates into the lesion, implantation of slow release PF4 conjugate formulations (biodegradable beads, etc.) or implantation of a subcutaneous reservoir bearing a catheter for directing the delivery of the PF4 conjugate to the specific location of the initial malignancy.

Formulations and dose will depend on the method of administration. A preferred administration method is the direct injection or instillation of a PF4 conjugate by a subcutaneous reservoir/catheter system that is limited to about 0.05 to 5.0 ml total volume in a single dose. A single dose may contain from 0.25 to 25 mg of PF4 (0.003 to 0.4 mg/kg) and may be administered as frequently as daily, wherein the patient receives one week of daily treatment per month, or three times a week, wherein treatment is administered continuously until significant patient improvement, or treatment may be administered as infrequently as one time per month. In certain situations, it may be necessary to administer PF4 conjugates by continuous direct administration over the course of several hours. Under such conditions, the volume is increased to permit slower administration of the desired dose.

Tumors of the brain can also be treated by adopting methods widely used in the treatment of gynecological and oral cancers, and are now applied with increasing frequency to brain and other cancers. Such methods, termed "brachytherapy," involve the delivery of locally high doses of radiotherapy (*Cancer, Principles and Practice of*

*Oncology*, Third Edition; DeVita, V.T., Jr., S. Hellman, S.A. Rosenber, eds.). Here, PF4 conjugates can be used in place of a radioactive agent. Implantable reservoirs for intracranial infusion of therapeutic agents are widely available and include the Omaya reservoir and similar devices. Implantation of the reservoir can be done at the time of initial surgery or later in a minor surgical procedure under local anaesthesia, by stereotactic methods.

B. Local treatment of unresectable lesions or recurrent lesions. Some brain tumors which are not resectable for technical and anatomical reasons may be treated directly, without prior surgery, with therapeutic benefit. Lesions which recur within one year of initial surgery are not generally appropriate for further surgical treatment and are treated by intratumoral methods. In this case, a preferred treatment is brachytherapy, which is described above.

Brain tumors can be treated as described above on an out- or an in-patient basis, as the patient's health permits.

The potential therapeutic benefits of PF4 conjugate treatment to the patient are:

(1) Restriction of tumor growth or indication of regression (as indicated by lesion imaging and/or biopsy).

(2) Improvement of symptoms associated with brain malignancy (incidence of headache, memory function, personality disorders, etc.) with or without observable effects on tumor progression.

(3) Increased time to postsurgical recurrence.

(4) Increased survival time.

Example 18 — Treatment of Kaposi's sarcoma, Psoriasis, Basal Cell Carcinoma, and Other Skin Disorders

Disorders of the skin can be treated intralesionally, wherein formulations and dose will depend on the method of administration and on the size and severity of the lesion to be treated. Preferred methods include intradermal and subcutaneous injection. Patients may be capable of self-administration. Preferred dosages are 0.05 to 5 mg PF4 per dose (0.7 to 70 mg/kg) contained within a volume of 0.1 to 1 ml. Multiple injections into large lesions may be possible, and several lesions on the skin of a single patient may be treated at one time. The schedule for administration can

be the same as that described above for the treatment of brain tumors. Formulations designed for sustained release will reduce the frequency of administration. Patients can be treated as out- or in-patients, as their health permits.

Example 19 — Systemic Treatment

Systemic treatment can be used for conditions such as small cell lung carcinoma, head and neck cancer, sarcoma, breast cancer, and colon cancer. PF4 conjugates can be administered by direct intravenous injection, or preferably by intravenous infusion lasting from 0.5 to 4 hours per single treatment. Patients can be treated as in- or out-patients. Patients may also be treated using implantable subcutaneous portals, reservoirs, or pumps. Multiple intravenous or subcutaneous doses are possible, and in the case of implantable methods for treatment, formulations designed for sustained release will be especially useful. Patients can be treated at dosages of 0.3 to 12 g of PF4 per period; preferably with 4 to 180 mg/kg in a volume of 60 ml to 2.5 liters per day.

A dosage is defined as a single dose administered as a bolus injection or intravenous infusion; or the compound can be administered to the patient as an intravenous infusion over a period of a day; alternatively, the compound can be administered in several bolus injections interrupted by periods of time such that the dose is delivered over the course of a 24 hour period. The most preferred method of treatment is to administer the compound to the patient in one injection or infusion per day.

Patients may be treated daily on alternative weeks for six weeks, or possibly for life. They may also be treated three times per week continuously, or they may be treated daily for life.

Example 20 — Regional Treatment

Regional treatment is useful for treatment of cancers in specific organs in the patient including, but not limited to, primary liver cancer, brain and kidney cancer, and liver metastases from colon/rectal cancer. Treatment can be accomplished by intraarterial infusion. A catheter can be surgically or angiographically implanted to direct treatment to the affected organ. A subcutaneous portal connected to the catheter

can be used for chronic treatment, or an implantable, refillable pump may also be employed. Patients can receive 0.05 to 1 g PF4 (1 to 20 mg/kg) in a volume of 10 to 400 ml per single dose. The schedule for treatment is the same as that described above for systemic treatment.

Example 21 — Lack of Toxicity of rPF4

In addition to human clinical trials, the lack of rPF4 toxicity has been demonstrated in reliable animal models. Specifically, a range-finding study investigated the acute toxicity associated with a single intravascular administration of a number of different test articles in mice. The general design allowed for two mice/sex to be administered a test article and be observed for 48-72 hours in order to determine the potential for acute toxicity. Based on the results of each dose level, the subsequent group of 2 mice/sex would receive either a higher or lower dose level of that test article. Mortality, clinical observations, body weight changes, and gross necroscopy findings were used to assess toxicity.

rPF4 was administered to 2 male and 2 female mice each at 100 mg/kg (Group 1) and 200 mg/kg (Group 2) and to 1 male and 2 female mice at 300 mg/kg (Group 3). Clinical observations revealed bruising at the site of injection in 1 male and 2 female mice. Body weight examinations revealed that there were no remarkable differences from dosing to termination. At necropsy, bruised tail was noted in one Group 1 male and two Group 3 females. In addition, bilateral enlarged preputial glands were noted in one Group 1 male, there were two foci on the left lateral lobe of the liver in one Group 2 male, the mandibular lymph node was enlarged in one Group 1 female, and there was a cyst on the ovary of one Group 2 female. Based on the incidence of the findings and a clear lack of dose response, it is believed that these findings are incidental to treatment (except for the bruising on the tail due to the physical trauma of the injection). Therefore, a single intravenous administration of 300 mg/kg rPF4 does not appear to have any adverse effects in mice.

In contrast, protamine sulfate was administered to 2 mice/sex at dose levels of 50 (Group 4), 25 (Group 5), and 40 (Group 6) mg/kg. Animals administered 50 mg/kg protamine sulfate displayed lethargy and rapid breathing, and one male and one female died shortly after administration. Based on the mortality, a dose level of 25

mg/kg was administered to the next group of 2 mice/sex. Both males displayed a ruffled haircoat postdose on day 1, but neither female appeared to be affected. Based on these results, the remaining two mice/sex were administered 40 mg/kg protamine sulfate intravenously. All four animals displayed rapid breathing and lethargy postdose, and one of the males also appeared to be sensitive to the touch. With the exception of one male administered 25 mg/kg, all other animals given protamine sulfate lost body weight when the terminal value was compared to the preinitiation value. At necropsy, one male administered 25 mg/kg was noted with enlarged bilateral preputial glands, one female administered 25 mg/kg had a cyst on the left ovary, and a bruised tail was noted in one female administered 40 mg/kg protamine sulfate. Based on the clinical observations noted, it would appear that a dose level of 25 mg/kg or more of protamine sulfate has severe deleterious effects on the mice.

Based on the results of the study, there is no overt acute toxicity associated with intravenous administrations of rPF4, whereas administration of 25-50 mg/kg protamine sulfate resulted in indications of toxicity or mortality in at least some of the mice.

Example 22 — Biodistribution of PF4 After Systemic Administration

Biodistribution characteristics are considered by those skilled in this art to be particularly relevant in establishing that a compound will have clinical utility—especially for systemic use. For an angiogenesis-inhibiting compound to have clinical efficacy, it must be possible to administer the compound at concentrations which will have the desired angiostatic effect at a particular physiological location without being toxic or causing any other deleterious effect. The biodistribution characteristics observed for PF4 further establish its clinical utility for the treatment of angiogenic diseases. Specifically, these results confirm the efficacy of systemic administration of PF4 to treat angiogenic diseases.

The biodistribution of rPF4 was assessed at three concentrations in both male and female rats. Perfusion of animals at 30 minutes and 24 hours following intravenous injection of $^{125}$-I-rPF4 and analysis of organ distribution of radioactivity demonstrated significant retention of counts within organ tissues at the 30 minute time point (Figures 1 and 2) which decayed substantially over the subsequent 24 hour

period. At the higher doses of rPF4, the relative distribution shifted from the liver, spleen, and kidney to include the lungs and other organs. Little radioactivity was found in the urine at the early time point, suggesting that the kidney-associated protein was not being eliminated by rapid filtration. At the 24 hour time point, however, substantial counts were detected in urine, suggesting that clearance in the urine may be linked to protein degradation.

The level of rPF4 found in the lungs (5-100 μg/g tissue) at the highest doses injected in these studies correlates with inhibitory activity of this agent on endothelial cells *in vitro*. In addition, this dose correlates with the level of rPF4 that effectively suppresses metastatic tumor growth in the lungs when the protein is administered systemically. In mouse tumor studies, a single injection of rPF4 is sufficient to suppress the growth of lesions in the lung, suggesting that the tissue residence of rPF4 is of greater importance in mediating the antitumor effects of rPF4 than is plasma level of the drug. Similar results are obtained when rPF4 is administered in a multidose subcutaneous regimen with the first injection delayed until 24 hours following tumor seeding, demonstrating that the effects of rPF4 can be induced by alternative systemic approaches.

Taken together, these data establish that the inhibitory effects of rPF4 do not require the long term exposure of endothelial cells to high plasma levels of the protein, but that local concentration of the protein by certain tissues leads to preferential inhibition of local neovascularization. This result further establishes the therapeutic utility of systemic administration of rPF4. In accordance with our invention, rPF4 conjugates will be useful as an intravenous infusion, or through other systemic administration approach, as a treatment for disseminated angiogenic diseases such as cancer, ocular neovascularization, and Kaposi's sarcoma.

Example 23 — Preferential Binding of rPF4 to Sites of Neovascularization -- Hamster Cheek Assay

An important aspect of the subject invention is the discovery that PF4 preferentially binds to sites of active angiogenesis. We have discovered that this unique binding property is retained even if PF4 is conjugated to another molecule. Another important aspect of the invention is the discovery that PF4 can be transported

systemically without loss of activity, and, advantageously, the PF4 will bind to, and accumulate at, sites of neovascularization. The advantageous binding and distribution properties of PF4 have been observed in a number of contexts as described throughout this specification. One compelling demonstration of the unique and advantageous properties of PF4 has been observed with systemically-administered rPF4. Specifically, the movement of rPF4 in hamster cheeks was used to evaluate the vascular transportation and binding characteristics of systemically-administered rPF4. In order to observe the movement of PF4, it was conjugated to a fluorescent label. For this assay, the rPF4 is conjugated to FITC as described above in Example 9B. The conjugation of rPF4 to FITC is done so that the location of the rPF4 can be readily visualized using cameras which detect the fluorescence emitted by the FITC-labeled rPF4. This experimental procedure is essentially as described by Rosengren *et al.* ([1989] *Microvascular Research* 38:242-254).

These experiments revealed that when fluorescently-labeled rPF4 is administered to a hamster and the vasculature of the hamster's cheek skin is viewed by intravital microscopy, distinct patches of bright fluorescence are detected in regions where angiogenesis is occurring. The binding of PF4 in this assay is not associated with leukocyte attachment to endothelium nor altered by the induction of local inflammation processes. These findings indicate that PF4 preferentially binds to endogenous receptors on proliferating endothelial cells.

Example 24 — Preferential Binding of rPF4 to Sites of Neovascularization -- Induced Angiogenesis Assay

Further evidence of the advantageous transport and binding characteristics of PF4 was obtained using a second experimental method which was adapted from the procedure described by Menger *et al.* ([1990] *Transplantation Proceedings* 22:821-822). The Menger *et al.* method involves the observation of the binding of PF4 in the vicinity of induced angiogenesis. In the assays reported here, the angiogenesis is induced by implanted islet cells from syngeneic hamsters. As in the hamster cheek assay described in Example 23, the PF4 has been labelled with FITC and administered systemically to the hamster. The binding of the FITC-labelled PF4 is then monitored, particularly in the region of the angiogenesis-causing agent. The fluorescently-labeled

PF4 is clearly seen to accumulate at the location of angiogenesis and move through, but not accumulate in, the surrounding vasculature. The results of this assay clearly demonstrate that PF4 preferentially binds to sites of angiogenesis. Thus, PF4 is highly advantageous for its ability to move through existing vasculature and ultimately accumulate at sites of active angiogenesis. The fluorescence associated with PF4 was monitored for hours and was found to be stably associated with the proliferating endothelial cells for periods of time clearly sufficient to facilitate accurate imaging of sites of neovascularization. The binding of PF4 to these sites was qualitatively and quantitatively higher than the presence of PF4 in vessels and tissues not involved in neovascularization.

Therefore, as described in detail herein, PF4 can, itself, be used as an effective angiogenesis-inhibiting compound which advantageously accumulates at sites of angiogenesis. Furthermore, because of PF4's preferential binding to sites of endothelial cell proliferation, a characteristic which is not lost by systemic administration routes or destroyed by conjugation to other entities, PF4 can be used to target second entities having desired properties to sites of angiogenesis. These second entities may have, for example, therapeutic or diagnostic utility.

Example 25 — Delivery of PF4 Activity to Specific Sites

For treatment of certain conditions, it is sometimes advantageous to direct biological activity to a specific location. For example, in order to inhibit solid tumor growth, it may be desirable to send PF4, or an analog with angiostatic properties, directly to the tumor site. This can be accomplished by coupling the PF4 (or analog) to an appropriate antibody, preferably a monoclonal antibody. The monoclonal antibody, which can be produced using techniques that are well-known in the art, will selectively seek out the target site. As the antibody moves to the desired location, it brings with it the PF4. Thus, the PF4 activity can be concentrated at a specific location.

General means of conjugating antibodies to polypeptides such as PF4 are well known to those skilled in the art and are discussed, for example, in U.S. Patent Nos. 4,671,958 (Rodwell *et al.*) and 4,792,447 (Uhr *et al.*). The PF4 may also be targeted to specific locations via analogous conjugation with binding proteins (e.g.,

thrombospondin or fibroblast growth factor), cell receptor molecules (e.g., CD4, lymphocyte function associated antigen-1 [LFA-1], and von Willebrand Factor [vWF]) or the complementary ligands, and non-proteinaceous biological effector molecules (e.g., ICAM-1, tumor associated-antigens, and prostaglandins).

For example, the monoclonal antibody, or other moiety, can be associated with PF4 at one or both pairs of lysine residues located near the carboxy terminus of PF4. It is preferable to modify less than all of the lysine residues. By associating a monoclonal antibody (or other moiety) at these residues, the angiostatic activity is retained while heparin binding is eliminated. Also, other amino acid residues may be substituted for the lysine residues before conjugation with appropriate moieties at these and other positions. Therefore, the compounds described here can be represented as follows:

```
                    F   G           H   I
                    |   |           |   |
A Pro Leu Tyr Xaa Xaa Ile  Ile  Xaa Xaa Leu Leu Glu Ser COOH
```

(SEQ ID NO. 11) where:

(a) **A** represents all or part of the polypeptide sequence consisting of residues 1 through 57 of PF4; **A** may or may not be present on said hybrid polypeptide;

(b) the designation "Xaa" represents any amino acid having a functional group suitable for covalent attachment; and

(c) **F, G, H,** and **I** are selected from the group consisting of monoclonal antibodies, polyclonal antibodies, fluorescein-isothiocyanate, fluorophores, toxins, cell receptor molecules, non-proteinaceous biological effector molecules, polyamino acids, polysaccharides, and chelators; at least one of the moieties designated **F, G, H,** and **I** must be present on said hybrid polypeptide.

In the above representation of the compounds described here, the vertical lines represent chemical bonding interactions as do the spaces between the amino acids on the horizontal line. The existence of specifically illustrated moieties associated at the